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Somatic embryogenesis response in Argentinian sugarcane genotypes for *in vitro* mutagenesis application

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Abstract Somatic embryogenesis is an excellent process for chemical mutation induction as somatic embryos originate from a single cell, hence limiting chimeras to very low frequencies in regenerated mutants. Callus formation and plant regeneration in sugarcane vary with genotype, explant type and culture conditions. Furthermore, optimization of the appropriate mutagen dose and dose rate is an important step in mutagenesis. We explored the somatic embryogenesis response of five genotypes developed and selected by the INTA sugarcane-breeding program, with the aim of identifying genotypes for successful *in vitro* mutagenesis. We evaluated the success rate of embryogenic callus initiation and proliferation, plant regeneration, acclimatization and field transfer. In addition, embryogenic calli of one genotype (INTA CP 98-828) were exposed to different doses of ethyl methanesulfonate (EMS) to induce genetic variation. Results showed different tissue-culture responses among genotypes. Four cultivars produced embryogenic calli and regenerated plantlets with high acclimatization and field survival. Cultivar INTA CP 98-828 showed the best *in vitro* culture response and was selected for mutagen treatments. In the mutagenesis assay, there were significant differences in recovery capacity, callus sensitivity and regeneration capacity among EMS doses evaluated. EMS concentrations ≤ 32 mM for 3 h exposure were optimal to regenerate a sufficiently large number of normal plants in INTA CP 98-828. Our results show the prospect of exploiting this approach to introduce new genetic variants in the INTA sugarcane-breeding program.

Key words *Saccharum* hybrids, callus, tissue culture, ethyl methanesulfonate

INTRODUCTION

The northern region of Argentina is favourable for the cultivation of sugarcane, and it is cultivated on an area of 376,223 ha, 73% of which is in Tucumán (Benedetti 2018). In this province, the sugarcane agroindustry is important because of its economic, social and environmental impacts, which contribute significantly to territorial development. Ever-increasing demand to expand the production of sugarcane leads to a continual need to develop new genotypes with improved agronomic characteristics. However, the high ploidy level, complex genome, narrow genetic base and poor fertility of modern cultivars render sugarcane genetic improvement difficult through conventional breeding (Lakshmanan *et al.* 2005; Rutherford *et al.* 2014). In this context, biotechnologies offer excellent opportunities for sugarcane crop improvement and these approaches are being integrated in worldwide breeding programs (Lakshmanan *et al.* 2005).

Plant tissue-culture methods are employed as important aids to conventional methods of plant improvement. Some *in vitro* conditions can induce heritable changes, called somaclonal variation, that can increase with treatment with a mutagenic agent (Rutherford *et al.* 2014). *In vitro* mutagenesis is an alternative tool to generate variability within the existing sugarcane germplasm. Mutant plants can then be selected for desirable traits in a breeding program.

In vitro regeneration through indirect somatic embryogenesis is the most utilized method in sugarcane and has well-established protocols (Snyman *et al.* 2011). Somatic embryogenesis in which embryos originate from a single cell is an excellent system for *in vitro* mutation induction as it reduces the risk of chimeras among regenerated plants (Ho and Vasil 1983; Suprasanna *et al.* 2012). Different types of embryogenic calli have been described for sugarcane cultivars (Ho and Vasil 1983; Taylor *et al.* 1992). Compact embryogenic callus, termed Type 3 by Taylor *et al.* (1992), appears to be the ideal subject for *in vitro* mutagenesis (Suprasanna *et al.* 2012). The genotype has a determining effect on the induction of somatic embryogenesis in many plant species, including sugarcane (Liu 1993; Snyman *et al.* 1996; Gandonou *et al.* 2005; Alcantara *et al.* 2014; Kaur and Kapoor 2016; Mittal *et al.* 2016; Sardar *et al.* 2016). Furthermore, plant regeneration capacity is specific and a genotype-dependent phenomenon and some sugarcane cultivars are recalcitrant (Sughra *et al.* 2014; Kaur and Kapoor 2016; Naz *et al.* 2017). In addition, the suitable mutagen dose and exposure time are important factors affecting mutagenesis that vary substantially depending on the plant material (Suprasanna *et al.* 2012).

Knowledge about the ability of sugarcane genotypes to produce embryogenic callus and *in vitro* conditions for achieving plant regeneration is required for the use of the *in vitro* mutagenesis method. Somatic embryogenesis on Argentinean cultivars developed by the sugarcane breeding program of National Institute of Agricultural Technology (INTA) has not been studied. Our objective was to determine the somatic embryogenesis response of INTA genotypes of sugarcane and adjust mutagenization techniques for the generation of genetic variability.

MATERIALS AND METHODS

Plant material

Five sugarcane cultivars developed (FAM 81-820, INTA CP 98-828, INTA NA 89-686, INTA NA 91-209) or selected (L 91-281) by the sugarcane breeding program of INTA in Tucumán (Argentina) were evaluated for their indirect somatic embryogenesis response. The genotype selection considered agronomic adaptation and performance of the cultivars. A control cultivar (NA 85-1602), which is suitable for *in vitro* culture, was included.

Establishment of cultures and induction of embryogenic calli

Cane tops were collected from 7-month-old plants at the Experimental Station Famaillá (INTA, Tucumán). Stalks were sterilized by ethanol 70% (v/v) and sodium hypochlorite solution 18 g/L under aseptic conditions. An immature leaf roll was excised, cut into thick discs (1 mm) and placed into culture medium MS3 (4.3 g/L; Murashige and Skoog 1962 (MS) basal salts and vitamins, 100 mg/L myo-inositol, 20 g/L sucrose, 0.5 g/L casein, 3 mg/L 2,4-D and 9 g/L agar Britania Lab, pH 5.8) with 0.4 g/L cefotaxime. Cultures were incubated in darkness at 28°C and sub-cultured biweekly.

Two protocols were tested to induce callus formation: (a) incubation for 8 weeks on MS3 medium, called MS3 protocol; and (b) 4 weeks on MS3 follow by 4 weeks on MS1 (same components as MS3 but with 1 mg/L 2,4-D), called MS3/MS1 protocol. Establishment capacity of the culture (EsC) was recorded after 1 week as the number of established explants/total number of explants × 100. Embryogenic capacity (EmC) was recorded after 8 weeks as the number of calli with embryogenic response/total number of calli × 100. Embryogenic callus, denominated as Type 3, was identified by its white, compact and nodular appearance. Calli were visually assessed according to percentage of Type 3 callus (PT3) of the total callus volume using the scoring system: 1 = 0-25%; 2 = 25-50%; 3 = 50-75% and 4 = 75-100% PT3. Score per experimental unit was calculated as the average value of total calli in the Petri dish. The experimental design was a completely randomized factorial (two induction protocols × six cultivars) with eight replicates per treatment.

Plant regeneration and acclimatization

Embryogenic calli were transferred to a regeneration medium (MR) that consisted of same MS3 components but with 5 g/L agar and without 2,4-D. MR was supplemented with 5 mg/L IBA (indole-3-butyric acid) every 4 weeks for root development. Cultures were incubated at 28°C in a growth chamber with a 16-h photoperiod. Regeneration capacity (RgC) was recorded after 12 weeks as the number of plantlets/dish. When plantlets reached 6-7 cm height, they were potted in a mixture of peat, sterile soil and perlite (4:2:1 v/v) and acclimatized in a greenhouse. Two procedures were investigated: (a) regenerated plantlets were transferred to transparent pots and were kept for the first week under high humidity by covering with thin plastic; and (b) plantlets were transferred to plastic seedling trays without covering. Acclimatization capacity (AC) was recorded after 2 months as the number of

acclimatized plantlets/number of regenerated plantlets × 100. The experimental design was a completely randomized factorial (two acclimatization procedures × five cultivars) with eight replicates of 10 plantlets per treatment.

The acclimatized plantlets were directly transplanted to a field in a completely randomized experimental design (60 cm plant to plant and 160 cm between rows distance) at Experimental Station Famaillá. Data for field survival (FS) were recorded as the number of surviving plantlets/total plantlets transplanted × 100.

Mutagenic assays

The embryogenic calli of cultivar INTA CP 98-828 were subjected to ethyl methanesulfonate (EMS) treatments following the method described by Sadat and Hoveize (2012) with modifications according to Suprasanna *et al.* (2012). Five treatments were evaluated: 0, 8, 16, 32 and 48 mM of EMS incubation with stirring for 3 h. Treated calli were sub-cultured for 6 weeks on MS3. Recovery capacity (RC) was recorded as the number of calli with embryogenic response/total number of treated calli × 100. Callus sensitivity (S) was determined by a modified Santana scale (Santana 1982): 1 - dead callus; 2 - callus alive but without somatic embryos; 3 - callus alive with small growth points with somatic embryos; 4 - callus growing in 50% of its volume with somatic embryos; and 5 - callus with normal growth and normal embryogenic capacity. The score per experimental unit was calculated as the average value of the total calli on the dish. Subsequently embryogenic calli was sub-cultured into MR for plant development. Regeneration capacity was recorded after 12 weeks. The assay was carried out in a completely randomized experimental design with 20 replicates with 15 calli for each treatment.

Statistical analysis

Generalized linear models (GLM) were used for data analyses. Percentage data of the variables EsC, EmC, AC, FS and RC were transformed to frequencies for analysis. Differences among levels of each factor ($\alpha = 0.05$) were tested by DGC (Di Rienzo *et al.* 2002). We used the statistical software package Infostat (Di Rienzo *et al.* 2017), with an interface with the software package R version 3.4.2 (The R-Foundation for Statistical Computing 2018).

RESULTS

Response of different genotypes to callus induction, regeneration and acclimatization

All genotypes showed high establishment capacity in culture, greater than 90% (Figure 1a). Nevertheless, EsC was significantly lower ($P < 0.0001$) for INTA NA 89-686 (92.56%) and FAM 81-820 (94.31%) cultivars than the control NA 85-1602 (99.13%). We observed browning of explants in most cultivars, with the highest levels in INTA NA 89-686 and NA 85-1602, although it disappeared during callus proliferation without adverse effects.

The five genotypes we used were able to form calli on medium supplemented with 2,4-D. Callus development started after approximately 7 days of explant culture, which was shown by the swelling of tissues and slight oxidation. After 8 weeks, three types of callus were clearly differentiated: (a) semi-translucent, non-embryogenic callus with a mucilaginous aspect; (b) friable and yellowish non-embryogenic callus; and (c) compact, whitish callus with surface globular structures (somatic embryos) known as Type 3 callus (Taylor *et al.* 1992). The abundance of different callus types was influenced by the genotype and culture conditions. The GLM analysis for the variable PT3 showed a significant interaction ($P < 0.0001$) between genotype and induction protocol (Figure 1b). Only the control and cultivar INTA CP 98-828 were similar for both the MS3 and MS3/MS1 protocols. INTA CP 98-828 showed PT3 greater than 75%, and INTA NA 89-686 and INTA NA 91-209 showed high PT3 as well as 50% of callus mass. The remaining portion of callus mass was mainly of the friable and yellowish type. Calli derived from cultivar FAM 81-820 were mucilaginous and did not regenerate into plants, so this cultivar was not used in subsequent culture steps.

The capacity to form embryogenic callus (EmC) ranged from 0 to 100%. Of the five INTA genotypes evaluated, four showed embryogenic capacity: INTA CP 98-828, INTA NA 89-686, INTA NA 91-209 and L 91-281. There was an interaction between genotypes and induction protocols ($P = 0.0252$) in producing embryogenic callus. The best genotype/protocol combinations were NA 85-1602 (control) with both MS3 and MS3/MS1 protocols, followed by INTA CP 98-828 with MS3/MS1 and INTA NA 89-686 with both MS3 and MS3/MS1 (Figure 1c). All these showed a high EmC greater than 90%. Moreover, INTA CP 98-828 (MS3) and INTA NA 91-209 (MS3 and MS3/MS1) gave a EmC greater than 75%, followed by L 91-281 (MS3 and MS3/MS1) as the least efficient genotype (~50%). FAM

81-820 did not form any embryogenic callus. Although genotypes vary in their embryogenic capacity following MS3/MS1 protocol, a better maturation of the somatic embryos was observed under magnification (40X) in all genotypes.

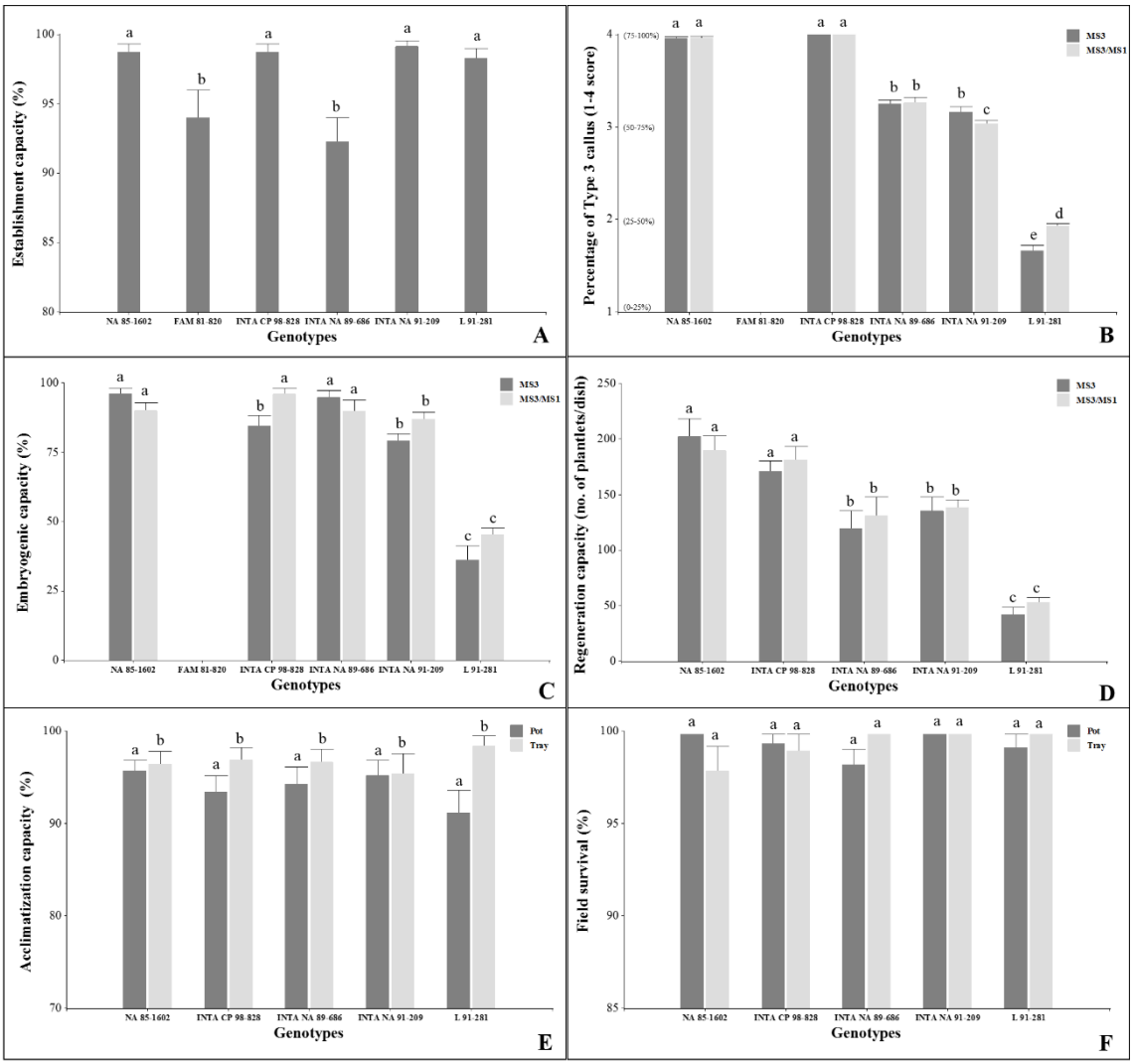

Figure 1. *In vitro* culture response of five INTA sugarcane genotypes, *via* indirect somatic embryogenesis. (A) establishment capacity, (B) percentage of Type 3 callus, (C) embryogenic capacity and (D) regeneration capacity of cultures, following two protocols of callus induction (MS3 and MS3/MS1). (E) acclimatization capacity and (F) field survival following two acclimatization procedures (Tray and Pot). Different letters (a-e) indicate significant differences (α = 0.05) by DGC test. Means ± SE.

Germination of somatic embryos was observed on the regeneration medium under light conditions. After 8-12 weeks, well-developed plantlets were observed in MR. The RgC was variable, with 7-17 seedlings (12 on average) being regenerated from a single embryogenic callus. The GLMs showed highly significant differences among genotypes ($P < 0.0001$). There were no significant differences among induction protocols ($P = 0.3151$) and there were no significant interaction effects ($P = 0.5887$). The genotype INTA CP 98-828, like the control (196 plantlets/dish), showed the best regeneration response (176 plantlets/dish), followed by INTA NA 91-209 (137 plantlets/dish), INTA NA 89-686 (125 plantlets/dish), with L 91-281 being the least regenerating genotype (47 plantlets/dish) (Figure 1d). Abnormal appearance, such as albinism, was not detected in any plants.

The regenerated plants were transferred to the greenhouse for acclimatization. In AC, there was no significant difference among genotypes ($P = 0.7972$), but there were differences among acclimatization procedures ($P =$

0.0145). All genotypes were successfully established in soil, with an AC greater than 90% (Figure 1e). However, the success of acclimatization was slightly higher on tray-culture (97%) than pot-culture (94%) procedures.

Regenerated plantlets were transferred to the field after initial acclimatization. There were no significant differences in field survival among genotypes, procedures and interaction between both factors ($P > 0.05$). For all genotypes, the field establishment was successfully achieved with 98% of plants acclimatized to field conditions (Figure 1f).

Considering all characters, INTA CP 98-828 was the most suitable for use in further mutagenesis studies under *in vitro* culture conditions. MS3/MS1 was the best callus induction protocol for this cultivar and the tray-culture procedure was the best acclimatization method.

Differential response to the mutagenic agent

INTA CP 98-828 was the best genotype yielding maximum embryogenic callus and regeneration as described above. However, the addition of a mutagenic agent produced different responses for embryogenic callus proliferation and number of regenerated plants.

The results of the GLM showed a statistically significant effect of mutagen dose on the recovery capacity (RC) ($P < 0.0001$) after 6 weeks. RC was different only at the 48 mM EMS concentration (Figure 2a), being 50% lower than for the control dose. There were no significant differences ($P > 0.05$) between the control treatment and 8, 16 and 32 mM.

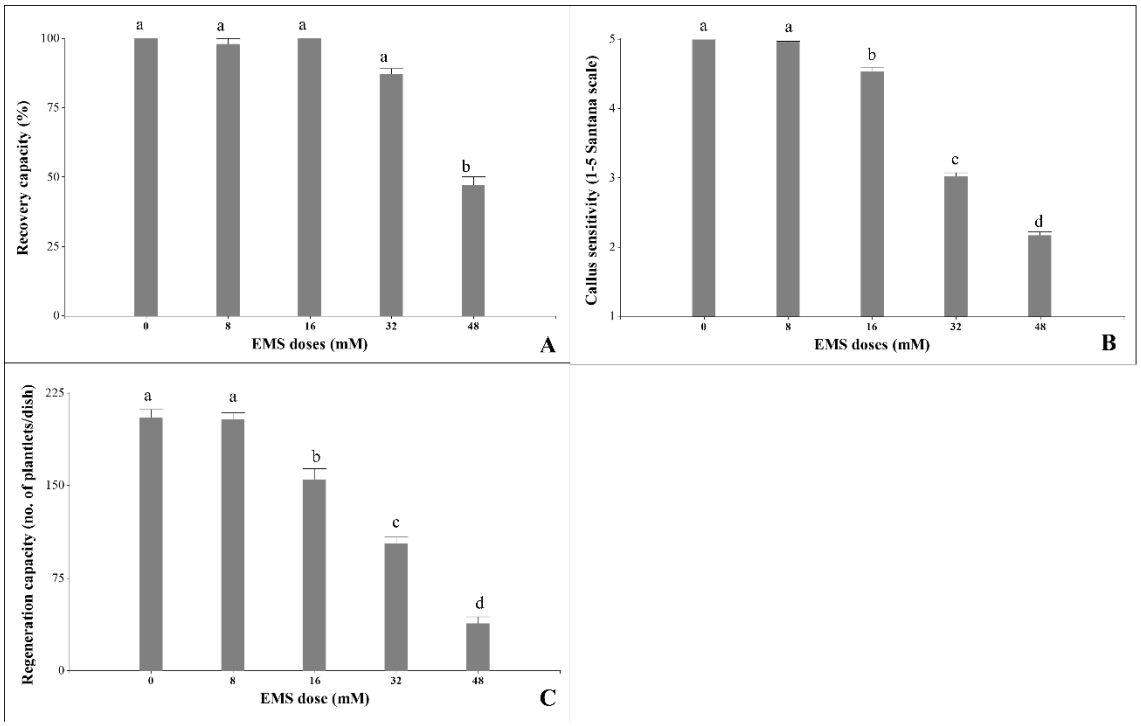

Figure 2. Effect of different EMS doses on (A) recovery capacity, (B) sensitivity and (C) regeneration capacity of embryogenic calli of sugarcane cultivar INTA CP 98-828. Different letters (a-d) indicate significant differences (α = 0.05) by DGC test. Means ± SE.

Analysis of the callus sensitivity (S) and regeneration capacity (RgC) showed that EMS-induced mutagenesis had a significant impact on callus proliferation as well as on plant regeneration. S and RgC decreased as the concentration of EMS increased (Figure 2b,c). The highest values for these variables were obtained at 0 mM and 8 mM concentrations, which did not differ significantly ($P > 0.05$), although differences occurred among the remaining EMS concentrations ($P < 0.05$). Calli with normal growth that greatly exceeded 50% of the initial mass (grade 5 in scale) and approximately 200 plantlets/dish were observed in both the 0 mM and 8 mM treatments.

The calli treated with a 16 mM dose increased more than 50% in volume (grade 4) and regenerated an average of 155 plantlets/dish. The 32 mM dose showed mainly wet and semi-translucent calli that did not proliferate in volume, but with some embryogenic growth points (grade 3) that regenerated 103 plantlets/dish. The highest dose (48 mM) exhibited the most severe effect. Most of calli were dark brown or black in color (grade 1) with cell death, or semi-translucent calli without mass growth (grade 2). However, this treatment also showed some calli with small embryogenic sectors (grade 3) that regenerated plants (38 plantlets/dish).

DISCUSSION

Somatic embryogenesis as a rapid, non-chimeric mode is preferred for the regeneration of plants for mutagenesis in crop improvement (Suprasanna *et al.* 2012), although this technique is constrained by low regeneration efficiency and strong effects of genotype-dependency. In our study, plants from five sugarcane cultivars were established by leaf-roll culture and were used for the successful induction of embryogenic tissues and subsequent plant regeneration. These cultivars were assessed for explant establishment, embryogenic capacity, number of green and albino plantlets, and acclimatization success involving callus induction following two induction protocols and two acclimatization procedures. As previous studies have reported in sugarcane, genotypes show high variability in their response to *in vitro* culture (Liu 1993; Snyman *et al.* 1996; Kaur and Kapoor 2016; Mittal *et al.* 2016; Sardar *et al.* 2016).

A range of explants have been used in somatic embryogenesis studies in sugarcane (Ho and Vasil 1983; Taylor *et al.* 1992; Liu 1993; Snyman *et al.* 1996; Sardar *et al.* 2016). Immature leaf cells are very prolific and thus are the preferred target tissue for rapid production of embryogenic callus (Lakshmanan 2006). We used immature leaf explants and obtained a successful establishment of *in vitro* culture. With the two protocols we tested, explants of all genotypes formed callus to varying degrees and different types of callus. Highly embryogenic (Type 3) callus was observed in four INTA genotypes that showed a differential response to induction protocols. Our results agree with other studies (Ali *et al.* 2007; Mittal *et al.* 2009) in which establishing highly embryogenic cultures genotypes depends on the genotype by culture environment interaction. Between the two induction protocols tested, a better somatic embryo maturation was observed from the MS3/MS1 protocol than with the MS3 protocol. Dibax *et al.* (2011) indicated that the reduction of 2,4-D concentration promoted embryogenic masses in Brazilian genotypes, in accordance with some of our observations. As Nadar *et al.* (1978) explained, the process of dedifferentiation and embryogenic-cell initiation required relatively high auxin concentrations, but lower auxin concentrations may be required for advanced embryogenesis stages.

The choice of the potential genotype that could be used in *in vitro* mutagenesis depends mainly on their capacity to regenerate plants. Although the germination rate of somatic embryos is very poor in most crops (Suprasanna *et al.* 2012), we obtained a considerable number of plants per genotype. In our cultivars, the ability to regenerate plants from callus cultures was largely influenced by the genotype. The capacity to produce regenerable calli from somatic embryos is also genotype-dependent in many species, including sugarcane (Kaur and Kapoor 2016; Naz *et al.* 2017). Our *in vitro* regenerated plants were successfully acclimatized under greenhouse conditions. Acclimatization in trays appears favorable for increasing stem girth and avoiding proliferation of fungi when compared with pot-culture conditions with high humidity. Furthermore, the regenerated plants responded well to field acclimatization. In all cases, the plant growth was vigorous, and the plant condition was healthy.

From our study, successful genotype and optimal *in vitro* culture conditions were identified that were used for *in vitro* mutagenesis experiments. Mutation breeding in crop plants is an effective tool for plant breeding, especially in crops that have a narrow genetic base. Chemical mutagens and irradiation have been used widely to induce a large number of functional variations in sugarcane (Rutherford *et al.* 2014) and others crops. Among chemical mutagens, EMS is the most commonly used in plants as it causes a high frequency of nucleotide substitutions, with the induction of new alleles due to point mutation within existing germplasm. By varying mutagen dose, the frequency of induced mutations can be regulated and saturation can be readily achieved (Waugh *et al.* 2006). EMS concentrations that we used (8–48 mM) are within the range tested for *in vitro* mutagenesis of sugarcane cited in the literature (Koch *et al.* 2012, Sadat and Hoveize 2012). In accordance with these studies, EMS concentrations ≤ 32 mM were optimal to regenerate a sufficiently large number of plants in INTA CP 98-828. The mutagenic treatments followed the similar protocol as Sadat and Hoveize (2012), but they were exposed to EMS for less time, indicating a higher sensitivity to EMS the genotype that we used. In addition, our results showed that most calli were recovered but the volume of embryogenic mass was affected after EMS treatment, and only embryogenic section regenerated complete plants. Our current work is focused on evaluating the regenerated INTA CP 98-828 plants that survived the mutagenic treatments.

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Réponse à l'embryogenèse somatique de génotypes argentins de canne à sucre pour une application de la mutagenèse *in vitro*

Résumé. L'embryogenèse somatique est une excellente procédure pour appliquer l'induction de mutations chimiques car les embryons somatiques proviennent d'une cellule unique, limitant ainsi les chimères à de très basses fréquences chez les mutants régénérés. La formation de cals et la régénération des plantes chez la canne à sucre varient selon le génotype, le type d'explant et les conditions de culture. En outre, l'optimisation de la dose de mutagène et de la durée d'exposition est une étape importante de la mutagenèse. Nous avons exploré la réponse de l'embryogenèse somatique de cinq génotypes développés et sélectionnés par le programme d'amélioration de la canne à sucre INTA, dans le but d'identifier les génotypes répondant le mieux à la mutagenèse *in vitro*. Nous avons évalué le taux de réussite de l'initiation et de la prolifération des cals embryogènes, de la régénération des plantes, de l'acclimatation et du transfert sur le terrain. De plus, des cals embryogènes d'un génotype (INTA CP 98-828) ont été exposés à différentes doses de méthanesulfonate d'éthyle (EMS) afin d'induire une variation génétique. Les résultats ont montré des réponses de culture tissulaire différentes selon les génotypes. Quatre cultivars ont produit des cals embryogènes et des plantules régénérées avec un taux d'acclimatation élevée et bonne survie au champ. Le cultivar INTA CP 98-828 a montré la meilleure réponse en culture in vitro et a été sélectionné pour les traitements par mutagène. Le test de mutagenèse a montré qu'il existait des différences significatives en termes de capacité de récupération, de sensibilité des cals et de capacité de régénération entre les doses de EMS évaluées. Les concentrations EMS ≤ 32 mM pour une exposition de 3 h étaient optimales pour régénérer un nombre suffisant de plantes normales avec INTA CP 98-828. Nos résultats montrent la possibilité d'exploiter cette approche pour introduire des variants génétiques dans le programme de sélection de la canne à sucre INTA.

Mots-clés: *Saccharum* hybrides, cals, culture tissulaire, méthanesulfonate d'éthyle

Respuesta a la embriogénesis somática de genotipos argentinos de caña de azúcar para la aplicación de mutagénesis *in vitro*

Resumen. La embriogénesis somática es un excelente proceso para la inducción química de mutaciones, ya que los embriones somáticos se originan a partir de una única célula, disminuyendo la frecuencia de aparición de quimeras entre las plantas regeneradas. En caña de azúcar, la formación de callos y la regeneración de plantas varían según el genotipo, el tipo de explante y las condiciones de cultivo. Además, la optimización de la dosis de mutágeno y el tiempo de exposición apropiados es un paso esencial en la mutagénesis. En este estudio, exploramos la respuesta a la embriogénesis somática de cinco genotipos de caña de azúcar desarrollados y seleccionados por el programa de mejoramiento genético de INTA, con el objetivo de identificar genotipos adecuados para la aplicación de mutagénesis *in vitro*. Se evaluaron las capacidades de establecimiento y proliferación de callos embriogénicos, regeneración de plantas, así como las capacidades de aclimatación y trasplante a campo. Así también, se expusieron callos embriogénicos de un genotipo seleccionado (INTA CP 98-828) a diferentes dosis de etil metanosulfonato (EMS) para inducir variación genética. Los resultados mostraron diferentes respuestas al cultivo de tejidos entre los genotipos evaluados. Cuatro cultivares produjeron callos embriogénicos y plántulas regeneradas con alta aclimatación y supervivencia en el campo. El cultivar INTA CP 98-828 mostró la mejor respuesta al cultivo *in vitro* por embriogénesis somática y se seleccionó para los tratamientos mutagénicos. En el ensayo de mutagénesis, hubo diferencias significativas en la capacidad de recuperación, la sensibilidad de los callos al mutágeno y la capacidad de regeneración entre las dosis de EMS evaluadas. Las concentraciones de EMS ≤ 32 mM con un tiempo de exposición de 3 h fueron óptimas para regenerar un número suficientemente grande de plantas normales en el cultivar INTA CP 98-828. Nuestros resultados exhiben la posibilidad de aprovechar este enfoque para introducir nuevas variantes genéticas en el programa de mejoramiento genético de caña de azúcar de INTA.

Palabras claves: híbridos *Saccharum*, callos, cultivo de tejidos, etil metanosulfonato